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Proteins and Their Peptide Motifs in Acellular Apatite Mineralization of Scaffolds for Tissue Engineering

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Many proteins in the inorganic/organic matrix of bone induce or modulate or inhibit mineralization of apatite *in vivo*. Many attempts have been made to mimic and understand this mechanism as part of bone formation, and ectopic mineralization and control thereof. Many attempts have also been made to use such proteins or protein fragments to harness their potential for improved mineralization. Such proteins and peptide motifs have also been the inspiration for attempts of making mimics of their structures and motifs using chemical or biological synthesis. The aim of this review is to highlight how proteins and (poly)peptides themselves impact mineralization in the human body, and how those could be used and have been used for improving apatite mineralization, for example, on or in materials that by themselves do not induce apatite mineralization but otherwise have interesting properties for use as bone tissue engineering scaffolds.

Introduction

With the aim of making new bone or replacing damaged bone, many venues have been chosen in the past. With the advent of ideas that aim to enable us to replace damaged bone without resorting to metallic implants, there is a need for materials and procedures that will comply with the requirements for clinical success. For bone tissue engineering applications, it is important to develop scaffolds that are both degradable and can induce apatite formation. The overall goal should always be to achieve the three O's of bone growth and repair: osteoinduction, osteoconduction, and osteogenesis.[1] Because hydroxylapatite self-induces further hydroxylapatite formation,[2–4] it may be a good starting point to coat the scaffolds with a thin layer of apatite for the scaffold to be successful. A large range of inorganic chemical modification (e.g., using silicate treatments) strategies drawing inspiration from the Nature for coating biomaterials with calcium phosphates *in vitro* have been attempted.[5] Indeed, it has been claimed that the essential requirement for an artificial material to bond to living bone is the presence of apatite or the formation of biologically active bone-like apatite layer on their surfaces,[6] although highly crystalline stoichiometric hydroxylapatite is probably not the best option for tissue-engineered constructs because of its low solubility.[7] One should note that cells bind to the implanted structures rather than implants binding to bone.[8]

However, there may not be enough autogenous bone or of inferior quality because of the health status of the patient to make this a viable option,[1] and since xeno- or allografts usually are advised against because of the risk of transmitting disease agents, the use of autograft is usually recommended for bone repair.[1] In this context, proteins or synthetic polypeptides have been suggested for induction of mineralization of constructs that are aimed at replacing damaged or weakened bone.

Because there are some differences in how the protein based mechanisms for acellular mineral nucleation and growth, many have suggested that there are two classes of inhibitors: those that affect nucleation and those that affect crystal growth.[9,10] One would expect that promoters in analogy also can be divided into two classes. Perhaps, surprisingly we shall note that some proteins can be either inhibitor or promoter depending on not only its state but also experimental parameters. For *in vitro* experiments this can also be influenced by experimental factors such as biopolymer and ion concentration, and pH. Thus, it is important to learn from the Nature how proteins and peptide motifs are used *in vivo* to induce nucleation and growth, and how they can be used to induce an appropriately formed apatite. In this review we will not focus on the cellular actions.[11–14]

Many proteins both in the extracellular matrix (ECM) and in blood will affect the mineralization as noted in the existing very wide and large literature. One would thus expect that it is very appealing to learn from the Nature how the mimics of the organic components in bone can be used to control mineral formation by learning how these modulate both the initial nucleation and the following crystal growth.

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This review aims to give a brief introduction to apatite and the proteins involved in mineralization followed by a more extensive account of how proteins themselves directly can influence mineralization of apatite and how that has been used to induce and modulate apatite formation for biomaterials and bone tissue engineering applications.

Apatites in Bones

There has been some debate whether the apatite in bone is hydroxyl apatite or carbonate apatite, although the apatite found in human bone is most likely poorly crystalline carbonated apatite with some hydroxyl character.[15–19] The inorganic phase of bone is apatite with different crystal size and substituents depending on when and where it is formed.[20] The first woven bone is biodegraded and replaced with lamellar bone that is more structured.[11,21]

It is not always clearly demonstrated which type of apatite is formed in the *in vitro* or *in vivo* experiments because often only the Ca/P-ratio, but neither the hydroxyl nor carbonate content, is determined. Thus, in this review the formed mineral in the referenced studies is in many cases simply referred to as apatite (at least when the determined Ca/P ratio is between 1.6 and 2). Below that the formed pure calcium phosphate may not be apatitic[15] but can be apatitic if Ca is partially substituted with Mg and other metals so that $(Ca + Me)/P > 1.6$.

The Proteins

Many proteins have multiple functions, so it is not surprising that a mineralization-related protein may have multiple functions in mineralization.[21–37] Overall, many of the mineralization-related proteins are acidic and phosphorylated. Some proteins contain dicarboxylic acids. Notably, dentin phosphoprotein contains 37% aspartic acid and 38% serine.[38] Three of four amino acids are Asp and PSer motifs such as PSer-Asp-PSer-Asp, Asp-X-Asp-Y, and PSer-W-PSer-Z, where X–Z may also be Asp or Ser.[39] And phosphoryns have multiple repeats of Asp-Ser-Ser[40] of which the serines often are phosphorylated. These findings suggest that alternating rather than homopolypeptides are important during mineralization in terms of calcium coordination, and apatite binding and modulation.

The differences in distribution of all these protein indicate that they have different roles in both space (specific tissues, see Table 1) and time,[20,41–45] because they are expressed with different timing during bone formation. But all proteins are not exclusively associated with bone; for example, biglycan and decorin are found both in bone and cartilage, although their glycosylation varies between tissues.[46] And others that prevent mineralization are not found in bone; for example, saliva contains statherin.[47,48] (See Table 1 for an overview of the tissue distribution of these proteins and a tentative summary of their roles in mineralization.) Collagen is the major protein in bone[11,21] but does not necessarily nucleate apatite *in vivo*.[49] Hence, one would assume that the noncollagenous bone proteins regulate bone mineralization[50] although the orientation of the collagen fibrils is important for the organization of the formed bone.[11,51]

Protein Binding to Apatite

Many proteins will bind from blood plasma to hydroxylapatite.[52] In fact, the wide difference in the ability of proteins to adsorb to apatite has been used in protein separation techniques since the 1950s.[53] The binding of proteins to apatite depends on their isoelectric point.[54,55] Clusters of carboxyl groups (rather than total amount of negative charges) strengthen protein binding to hydroxylapatite.[55] The dicarboxyl on Gla in bone Gla protein makes it bind stronger to apatite than to amorphous calcium phosphates.[56] Gla has also been shown to be vital for osteocalcin and oligopeptide mimic to bind to apatite.[50,57,58]

For N-terminal fragments of statherin, it is the sequence, conformation, and the total charge rather than the chemical groups that are important for its binding behavior to apatite.[48] The sign of the charge is also important as demonstrated by the higher affinity[48,59] to and lower elutability[60] from apatite of negatively charged proteins compared to positively charged proteins (at physiological pH).

A number of amino acid sequences with three carboxyls and/or phosphates have been identified and stipulated to have a role in the binding of phosphoryn to apatite.[61] A Glu-rich region in osteonectin and bone sialoprotein and Gla in proline-rich salivary proteins have been suggested as being responsible for the binding to apatite.[62–64]

On the other hand, for some apatite-binding proteins only one or two phosphoserine residues are enough to increase their adsorption on apatite.[65] But it has been noted that at least three amino acids are necessary to span one apatite unit cell.[62] Just the presence of a long charged sequence does not necessarily mean that it is readily available for binding of calcium for either apatite nucleation or protein binding to apatite. The conformation of the polypeptide or more specifically the structure of the carboxylated and/or phosphyrolated amino acids could be just as important.[40] Part of the function could be lattice matching between mineral and polymer,[66–69] which is supported by data on alternating arrangements of charged domains on enamel crystal surfaces.[70] Note the dimensions of the unit cell of HA: $a = b = 9.4$ Å, and $c = 6.9$ Å.[69]

Similar results were recently noted from X-ray diffraction (XRD) data on porcine osteocalcin that yielded a good periodic match between five Ca ions bound at three Gla at consecutive α-helix turns and Ca positions at the face parallel with the c-axis of hydroxylapatite.[57] A synthetic oligopeptide mimicking this Gla containing α-helix yielded good binding at the (100) face of hydroxylapatite, which was strongly dependent on the γ-carboxylation of Glu.[58]

Also homopolypeptides such as poly-Glu can be used to induce nanocrystalline apatite.[71] However, as already mentioned, many phosphoproteins use alternating peptide sequences for binding to apatite and modulating apatite growth. This, again, stresses the role of conformation of polypeptides in relation to mineralization. The dimensions of the formed β-sheet are important because it probably is better if it matches the lattice dimensions of the mineral surface that is to be encouraged.[72] For example, the periodicity of poly-Asp is 6.7–6.9 Å and would fit the c-direction of the (100) surface of octacalcium phosphate (OCP) (6.87 Å). Also, the modeling of binding of oligopeptides to apatite or OCP indicated a better fit for β-strands comprising PSer-Asp or Pser-Pser-Asp rather than just repeats of either PSer or Asp.[67] This could point at differences in the roles of electrostatic interaction and chelation in mineralization. But then carboxylated β-sheets can be induced or stabilized by Ca^{2+} binding[73] or adsorption to apatite.[58]

Table 1. Summary of the Described Roles of the Major Proteins in Acellular Mineralization *In Vitro* and *In Vivo*, Their Main Suggested Motifs for Acellular Mineralization and Tissue Localization, and References

Protein	In vitro *adsorbed*	In vitro *dissolved*	In vivo	*Suggested motif*	*Tissue*	*References*
Albumin	Nucleation, sub dep Dissolution?	Inhibit seeds, conc dep	Inhibitor, inhibit seeds in saliva		Blood and all with blood contact	90, 96, 110, 115, 121, 125–130
Aggrecan–proteoglycans	Inhibit? Promote?			Sulfated glycosylation	Cartilage	34, 41, 43
Amelogenin	No effect	Promote, elongate, conc dep	Nucleate and organize apatite	TKREEVD	Enamel	20, 128, 131, 132
Biglycan/decorin	Nucleator Glyc dep	Inhibit Glyc dep	Collagen organization, nucleation	Chondroitin or dermatan sulfate	Connective tissue, bone, teeth	41, 43, 44, 46, 110, 113, 133–138
Bone acidic glycoprotein 75			Nucleator, promoter	(DE)7	Bone and dentin	20, 63, 139
Bone sialoprotein	Promote conc dep	Inhibit seeded but not nucl.	Promote	E8, E6, E4	Bone and dentin	20, 27, 63, 84–86, 109
Collagen type I	No effect	No effect	Structural		Bone, cartilage	11, 24, 51, 116
Chondrocalcin (collagen type II)	No effect on nucleation	Inhibit nucleation			Bone, cartilage	27, 85
Fetuin		Inhibit nucleation	Inhibit	DDXBXE, BXEXDXXE, B = E or D	Blood	140, 141
Fibrinogen	Sub dep				Blood	98
Fibronectin	Inhibit seed growth	Induce *de novo*, grow seeds	Nucleate		Blood	96, 127, 142
Matrix Gla protein			Inhibit ectopic	Gla, ESHESMES[a]	Cartilage, blood, arteries	20, 42, 100, 143
Osteocalcin = bone Gla protein	Nucleator	Inhibit *de novo* and seed	Turnover, inhibit	Gla, ESS,[a] EGSE[a]	Lamellar bone, cartilage, arteries	16, 20, 27, 50, 56, 63, 110, 144, 145
Osteonectin	No effect	Inhibit seed but not nucleation		EETEEE	Bone, dentin	27, 50, 62, 116
Osteopontin	No effect on nucleation	Inhibit nucleation but not growth	Inhibit ectopic, turnover, formation.	Regions rich in pS, D, and E	Bone, arteries etc.	20, 27, 63, 85, 101, 102, 146, 147
Phosphoproteins (phosphoryns, dentin sialo-phosphoprotein)	(Weak) nucleator, promoter, sub dep, conc dep	None to weak inhibit and nucl. and seed, conc dep	Nucleate, minor promoter	Region w only: (DSS)3-14 with N at D, (pSD)2-3, DXDY	Extracellular matrix, dentin	27, 34, 38–40, 61, 84, 85, 94, 110, 123, 148
Statherin	Inhibit formation and dissolution	Inhibit	Inhibit nucleaton and growth	DpSpSEE	Saliva	48, 103, 104, 115
Vitronectin	Inhibit seed growth	Promote nucleation			Bone, blood	96, 149, 150

The *in vitro* results have been grouped into "adsorbed" and "dissolved" to reflect the experimental setups. Of course any dissolved protein will also be adsorbed. The results regarding albumin are a bit contradictory, and the results are from a large set of different experimental setups, but can in part be explained by differences in concentration.[9] See more in the section Adsorbed versus Dissolved. "Sub dep" indicates that the substrate surface and the protein conformation play a role for when (pre)adsorbed.[34,98,99,151] "Glyc dep" indicates that the action depends on the glycosylation, and that few dermatan sulfated proteins do not induce nucleation.[133] "Conc dep" indicates that the effect depends on the concentration of protein in solution, especially when the results come from hydrogel studies. Generally, a higher protein concentration impedes nucleation or growth,[9] just as preadsorption might hinder seed growth. See more in the section "Concentration of Protein."

Then follow columns on the reported roles *in vivo* and the suggested mineralization related motifs. A "p" implies possible phosphorylation of the following amino acid.

[a]Plausible includes D, E, or S. Some S maybe pS, but that varies between proteins and location. X, Y, W, and Z indicate any amino acid. For dentin (sialo/phospho)protein most often it is S or D. A number after an amino acid (sequence) indicates number of repeats. See for example, Amino acid. (February 8, 2008). In *Wikipedia, The Free Encyclopedia*. Retrieved 15:42, February 8, 2008, from http://en.wikipedia.org/w/index.php?title = Amino_acid&oldid = 189968304 for a description of the amino acids and their abbreviations. Different sources gave different motifs for fetuin. For some proteins the online database Human Protein Reference Database (http://www.hprd.org) was used.[152]

In the second last column the tissues in which the proteins have been found are mentioned. In the last column are the references for each protein. Absence of information in any box only implies just that. Note that some of the attributed roles of the mentioned proteins are not yet consensual, so some precautions should be taken before drawing any extensive conclusions from this summary table.

It is also important to note that apatite is known to activate both the coagulation[74,75] and the complement[76,77] systems. On the other hand, activation of bodily defense systems via the complement and coagulation systems has been implied to play a positive role in osseointegration.[78]

Proteins and Apatite Formation

The organic components of the ECM are important for both starting the mineralization by acting as seeds, and control of the growth of the crystals by influencing the density of seeds, and the orientation and organization of the formed crystals[79,80] or acting as inhibitors.[81] In many cases they also contribute to the strength of and stabilize the mineralized tissue.[79] Apatite crystals have been detected in the grooves and channels of collagen type I.[51,82] Apatite does not normally form on collagen by itself *in vivo* although one can grow minerals on it *in vitro*.[83] Further, the carbonate apatite structure is tuned by the organization of the collagen type I network.[24,51]

The accumulation of (bone and dentine) sialoproteins in bone and dentine suggests that they have a role in the apatite mineralization *in vivo*.[63,84] Bone sialoprotein is highly phosphorylated and rich in polyglutamic regions. Dentin sialoprotein is similar but is low in phosphorylation. Bone sialoprotein is an effective apatite nucleator *in vitro*[84,85] in hydrogels, whereas dentin sialoprotein has a much lower affinity for apatite and only has a small effect on nucleation and growth.[84] Glutamic acid (Glu)–rich regions have been attributed to the nucleation properties of bone sialoprotein.[86] As reviewed by Boskey, phosphophoryn and bone sialoprotein can be both nucleators and inhibitors[28] of apatite mineralization.

Osteocalcin adsorbed in the surface region of collagen/apatite composite or apatite alone can switch on the remodeling cascade.[87–89] Osteocalcin present in solution (and thus also on the surface) inhibits the nucleation and growth of apatite crystals *in vitro*,[27,50,65] and the presence of Gla is crucial.[50] But osteocalcin is probably more important for remodeling than for nucleation[45] due to its late appearance during bone formation.

Preadsorbed dentin matrix protein 1 induces apatite formation on glass plates *in vitro*.[90] Some domains undergo a shift to β-sheet conformation upon Ca binding that precedes mineralization.[91] The β-sheets are also proposed to act as templates for mineralization.[73] It would seem that the Ca ion binding also acts as an electrostatic shield that is important for this change in conformation.

Phosphophoryn in the mineralized dentin matrix is highly phosphorylated.[40] It is believed to be involved in the regulation of the mineralization processes by binding to collagen, nucleating mineralization, and controlling crystal growth,[40,61,92–95] and inhibits seeded growth.[34]

There are many proteins that are not specific for bone tissue that also take part in the formation of bone especially if they are present in the blood stream. Fibronectin and vitronectin modulate and induce *de novo* growth of apatite crystals when adsorbed, but inhibits apatite growth when adsorbed on already formed apatite.[96] Apparently, adsorbed fibrinogen and albumin may have different effects on mineralization depending on the substrate[97,98] that perhaps differently affect their conformation upon adsorption and also their ability to affect mineralization. The type of seed crystal has shown to be important when phosphoproteins are dissolved.[34] This could be due to differences in ability to adsorb to the surfaces. Secondary structure of a protein can be important for its ability to induce apatite formation after adsorption,[61,99] perhaps by providing a stiff template or chelator as starting point for nucleation.

Most of the reported posttranslational modifications that impact on the mineralization modulation ability of proteins are covered in the Supplemental Material. Overall, most of the proteins that induce or inhibit apatite nucleation and/or growth contain clusters of negatively charged groups like sulfate, carboxyl, and phosphate that act via electrostatic interaction to attract or coordinate calcium ions at apatite surfaces, in solution and adsorbed.

Ectopic mineralization

Mineralization in soft tissue is in most case not functional and can be lethal especially if it persists in the arteries. So an active control seems to be of vital importance. To curb ectopic mineralization there are a number of control proteins such as matrix Gla protein,[42,100] osteopontin,[40,101,102] and statherin.[48,64,103,104] The sulfated saccharides on soft tissue proteoglycans have been suggested to play the role as inhibitors although the mechanism still is not clear. Also, as mentioned earlier serum and albumin can delay and/or inhibit nucleation and growth.

Bone Gla protein and osteonectin, but not the Ca^{2+} binding proteins calmodulin and parvalbumin, inhibit *in vitro* growth of hydroxylapatite.[50,100] It was suggested that the conformation for binding to apatite was not favorable for the latter two proteins. Thus, Ca^{2+} binding by itself may not always be a good marker for which proteins control apatite formation. Notably, negatively charged amino acid motifs are important in both ectopic and topical mineralization.

Adsorbed versus dissolved

Experimental models with either dissolved or adsorbed proteins have been used to elucidate if certain proteins act as inhibitors or promoters of mineralization. In many cases proteins that act as inhibitors of apatite nucleation and growth when dissolved also act as promoter of mineralization when adsorbed.[16,22,85,98,105–113] But for the case of bone and dentin most of the matrix proteins are not in solution.[34,114] It has been suggested that a difference in conformation for proteins as dissolved and adsorbed can account for some of the observed differences in behavior with regard to apatite formation.[50] Preadsorption of proteins could lead to induction of mineralization if the mineralization-modulating proteins are in an appropriate conformation. Further, some proteins probably can bind to already formed mineral nuclei and thus stabilize them for further growth,[16] inhibit dissolution,[115] or inhibit further growth.[104]

The adsorbed proteins may in different ways affect mineralization caused by the change in interfacial energy after the protein adsorption. It has been suggested that proteins when adsorbed on any surface can promote nucleation, but when adsorbed on already formed crystals they may decrease further crystal growth.[96,105] It has also been suggested that nonspecific protein adsorption decreases the substrate–liquid surface tension and thus lower the nucleation ability of

the substrate surface[9] but might mostly be an effect on non-inducing proteins covering the surface.

Concentration of protein

Along the *in vivo* timeline of mineralization, the presence of proteins will change also their concentration. Protein-induced mineral nucleation in solution decreases the amount of free ions and thus can drive the solution below (super) saturation and thus affect the ability of already started apatite formation to grow further. In the same manner Ca^{2+}-chelating proteins in solution will increase the dissolution of already formed apatite crystals. Although some proteins can bind calcium when dissolved, it is also possible that their adsorption on specific apatite faces will inhibit or direct apatite growth.[116]

Apatite mineralization *in vitro* is increasingly inhibited with an increasing concentration of serum solution, most likely because the adsorbed proteins block further growth[117–119] but can also lead to differences in morphology of crystal coatings after mineral growth on a variety of calcium phosphates.[120] Human serum albumin has been pointed out as an important factor in this process,[118] but also the biopolymers that bind to different sites on apatite are more effective together at inhibiting apatite growth.[121] The effect of proteins on apatite growth and transformation depends not only on the proteins but also on the seed crystals. Overall, serum proteins are assumed to impede *de novo* formation of apatite but may not always hinder seeded growth.[122]

For many mineralization-related proteins, it has been found that they induce growth when dissolved in small amounts, but at a certain point they start to inhibit further growth of hydroxylapatite.[27,63,84,123,124] Each of these *in vitro* effects has been attributed to interactions between the protein and specific faces of apatite. Combes and Rey have proposed that at low protein concentrations nucleation and growth are not much inhibited, but at higher concentrations adsorbed protein will inhibit growth (rather than nucleation) to such an extent that the overall mineral formation is inhibited.[9]

Proteins and Polypeptides in Making Mineralized Constructs

There is a very extensive body of research aimed at making use of oligo- or polypeptides for modulating cell attachment and growth, but so far the specific use of (poly)peptides aimed at acellular induction and modulation of apatite formation for use in bone tissue engineering applications is more sparse. In the following sections, we will highlight and comment on some more recent results from the use of proteins and peptides for making mineralized biomaterials and biomaterials that promote (acellular) mineralization.

Blends of Mineral and Proteins

There is a vast body of research on mixing proteins or peptides with apatite particles to make mineralized biomaterials. The aim is not necessarily to impose properties on the apatite particles themselves but rather to make composites in which apatite particles are immersed in an organic matrix, often in an attempt to mimic bone matrices, but very often not aiming to use the ability of the polypeptides to induce or modulate mineralization.

The success of composites of fibrin sealant and ceramic *in vivo* has been reported to be ambiguous, but in some cases they were able to induce the formation of woven bone.[153] Because the bone remodeling will transform the woven bone into lamellar bone,[11] it might be of interest to aim at making scaffolds that at first induce formation of woven bone *in vivo* rather than lamellar bone.

There have been some studies of making collagen–mineral composites[154–156] aimed at inducing bone repair. This type of matrix can readily be loaded with growth promoters for a wide variety of cells. This approach also benefits from the seeded growth *in vivo* that is less susceptible to interference from adsorbing proteins with regard to crystal growth. The main drawback is the nonautograft collagen with all the accompanying issues regarding disease transmission and immunorejection. The use of synthetic and biodegradable polymers that would provide a 3D network similar to that of collagen would hopefully circumvent those problems.

In a recent study of a collagen hydrogel aimed at soft tissue repair, it was suggested that calcium phosphate should be included[157] because it has been suggested that Ca^{2+} ions are necessary for neurite growth.[158] Thus such ions released from the scaffolds also would improve neurite healing.[157] Indeed, a premineralized structure perhaps could also act as a reservoir of ions to promote mineralization. Also, it has earlier been suggested that because elevated ion concentrations are needed for apatite formation, dissolution of already formed or coated apatite will help to maintain the local ion concentrations at elevated levels.[159]

Hydrogels

A synthetic self-assembling oligopeptide (Ace-Gln-Gln-Arg-Phe-Glu-Trp-Glu-Phe-Glu-Gln-Gln-NH2) that forms β-sheet fibrils has been shown to promote healing of dental lesions. Two potential nonexclusive mechanisms were proposed; the peptides form a gel that acts as Ca ion chelator and/or it acts to inhibit apatite dissolution in the acidic oral environment.[160] This study points to the possibility of repairing small defects in mineralized tissues with a gel structure that itself is not initially mineralized. To some extent this has already been achieved without polypeptide gels when the damaged section of bone has been delimited by a membrane barrier,[161,162] but gelatin foams might impede healing by obstructing bone marrow growth[163] although the authors comment on the possible use of bone inductive components to overcome this problem for large (even critical sized) bone defects.

The hydrophobic and basic P15 sequence (GTPGPQGIA GQRGVV) from collagen has been shown to bind to apatite particles. P15-coated apatite particles better induce bone formation when immersed in hydrogels and injected *in vivo* than just the apatite.[164,165] This again stresses the usefulness of (synthetic) oligopeptides, but also when load bearing is not an immediate essential requirement that injectable hydrogel/apatite composites could have a positive effect on bone formation.

Polypeptide Coatings

There are some approaches to drug delivery that make use of calcium phosphates; the drug is either present during the mineral preparation[166,167] or later adsorbed onto the

mineral[168–170] and also bone morphogenetic protein (BMPs).[171] One would expect that the former approach will enable a higher load and also a prolonged release. One could possibly alter the release kinetics by adding polypeptides to the outer surface of the apatite particles to make the mineral degrade slower or with a delayed onset of dissolution. This shows that the mineral phase of tissue engineering constructs also could be used as drug delivery devices. One study showed prolonged drug release by coating the mineral particles with albumin,[170] but it is not clear if it is due to prolonged release of the adsorbed protein or because the mineral is degraded slower, although it has been shown that proteins adsorbed to apatites will delay their dissolution.[103,115]

Electrodeposition of amelogenin promotes ordered apatite mineralization *in vitro* and has been suggested as a way to produce mineralized constructs.[172] Perhaps it would be interesting if this approach could be translated *in vivo*, that is, for repair of decayed enamel by using a chimeric approach with one end binding to the existing enamel and the other inducing enamel repair.

Further, as noted in a recent review, phosphorylation of synthetic polymers could be a way to mimic phosphorylated proteins in how they promote mineralization.[173] This would avoid much of the problems associated with allogenic proteins whilst being applicable to a wide variety of polymers. Although one would again expect that conformation is an important issue to assure proper mineralization.

Proteins Coprecipitated with Mineral

As noted above there is a difference between coating an apatite and having the organic phase incorporated during the fabrication of the apatite itself, either as particles or coating on devices. By having polypeptides or proteins present during mineralization *in vitro*, it is possible to affect the morphology of the formed apatite.[174–177] In many cases this type of incorporation of proteins or peptides into an apatite matrix will make the apatite less crystalline and thus also more susceptible to degradation by dissolution, although the mechanical strength could be improved by incorporating proteins.[177] Apatite in bone is more easily dissolved than synthetic hydroxylapatite.[7,178,179] Perhaps this is an desired starting point because the aim is to have the mineralized scaffolds being subjected to bone remodeling, which includes resorption of the initial apatite.[180] To some extent this has been achieved by a matrix of collagen and apatite nanoparticles that was shown to induce processes of bone degradation and formation that is similar of bone remodeling [176,181,182] although the formed bone was not hierarchical.

By adding a phosphoprotein to collagen, one can induce apatite formation *in vitro*,[183] but because of the nonautograft-related problems, it might be better to aim for recombinant collagen.[83] However, the incorporated protein could improve the mineral phase by conveying a function such as improvement of degradation kinetics of the polymer substrate[174,175] or adherence or stimulation of bone forming cells[184,185] (see also above paragraphs on drug delivery in the Peptide Coatings section).

Even much simpler molecules such as peptides and polypeptides have been used to form apatitic constructs. Coprecipitation with monomeric peptides has been shown to have potential to make apatite nanoparticles that in turn can be mixed with dextran sulfate to make 3D sponges. Aspartic acid was shown to be more successful than both arginine and lysine in making the sponge and getting the best cellular response to the hybrid although the scaffold was deemed too weak to be suitable for load-bearing applications.[186]

Acidic peptides have been combined with hydrophobic groups (peptides or alkyls) to produce self-assembled nanofibers to be used for inducing mineralization,[187–189] perhaps as a new class of injectable systems[69] that perhaps are better suited for small rather than critical-size defects.[190] Nanofibers made with amphiphiles with alkyl groups in the hydrophobic tail and serine or phosphoserine near the hydrophilic terminal gave amorphous deposits and apatite, respectively.[189] Notably, in the latter case the crystals were oriented along the fibers. A similar system with phosphoserine promoted apatitic mineralization when coated on titanium.[191] Amphiphiles with only peptides with aspartic acid in the hydrophilic tail that also forms nanotubular fiber meshes[192] could probably also act as an organic matrix for mineralization.[193]

Chimeric Peptides

Some effort has been made to make protein or oligopeptide coatings on either apatite particles or apatite coatings to enhance cell response. Synthetic polypeptides that use specific functionalities from more than one parent protein could be used in tissue engineering applications for inducing bone formation.[194] For example, one part binds to already formed apatite and the second is used as a cell-binding ligand. Indeed, many of the apatite-binding proteins also have domains with an arginine-glycine-aspartic acid (RGD) or other cell-binding ligands.[194] It has been suggested that by combining apatite-nucleating domains from one protein and cell-binding domain from another, one can induce desired function in biomedical applications.[126] The apatite-binding domain from pig bone sialoprotein was combined with the collagen-binding site of decorin into a chimeric protein. This chimer was bound to collagen and was found to induce apatite formation.[126] Gilbert *et al*. used a similar approach using the N15 terminal from statherin and an RGD containing hexapeptide from osteopontin,[195] which mediated a dose-dependent adhesion of melanoma cells but not without the hexapeptide. An even more downscaled approach is to attach an RGD sequence to a septa-glutamic acid.[196] The glutamic acid residue bound to the apatite, and the RGD sequence attracted mesenchymal stem cells more effectively than the RGD without the septa-Glu. A recent study has cast a doubt on this type of approach because it was claimed that since apatite is so prone to protein adsorption, any preattached cell ligands will be covered by an array of blood proteins,[197] although the approach still could be useful to improve cell seeding prior to implantation.

There already are chimeric proteins in the body because many of the bone matrix proteins bind to collagen and have sites for apatite formation or osteoblast attachment—for example, osteonectin[49] and bone sialoprotein.[198,199] The use of synthetic chimeric polypeptides could very well be a way to avoid the xeno/allograft–related problems while tailoring specific polypeptides for tissue engineering approaches. In the case of bone tissue engineering, the chimeric approach might be useful for coating or loading of *ex vivo* formed apatite constructs.

Concluding Remarks

The remaining crux is to determine if protein–apatite (coated) scaffolds can have a positive effect on apatite formation *in vivo* under the interference from blood proteins and surrounding tissue, and ultimately lead to new bone formation and remodeling in the implantation site. The polymeric scaffold that is intended as a template for apatite formation does not necessarily have to fulfill the ratios of mineral-to-organic phase of mature bone because the aim of tissue engineering is to create a starting point for the human body so that remodeling or healing is promoted. Ideally, the polymeric peptide (coated) scaffold should also be tailored for angiogenesis and a host of other characteristics.

Proteins are natural and necessary components of the mineralization of bone apatite *in vivo*. Taking the step of using (synthetic) proteins or protein fragments to induce apatite formation is thus very appealing. Such elements could be used in the preparation of scaffolds for enhancing the apatite mineralization process that leads to bone formation.

Because there are so many proteins that affect the mechanical, biochemical, and physicochemical properties of the formed apatite, by using proteins or well-designed fragments it should be possible to

- induce nucleation,
- induce and modulate growth,
- incorporate polypeptides or proteins during apatite growth that yield properties that can induce bone remodeling and vascularization, and
- coat the apatite with polypeptides that induce appropriate cell attachment and remodeling, and modulation of mineral dissolution.

The idea is thus to make use of a combination of strategies to achieve a good starting point for the bodily responses to take over so that the engineered scaffold can be transformed into mature bone.

For the future we already now see an increasing trend in borrowing traits from various mineralization-related proteins (rather than the whole protein) and applying them for tailored mineralization on scaffolds that should lead to mineral phase for its intended function or for such that mineralization is better avoided. One would perhaps also expect more computer modeling of existing apatite-binding domains to determine more completely how proteins interact with apatite surface and ions in solution, and compare those findings with structural matching between apatite and novel oligopeptides. Current trends and advances in oligo- and polypeptide design and synthesis promise a very high degree of control of end product that in the end could be useful in improving the mineralization characteristics of emerging multifunctional bone tissue engineering scaffolds.

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Disclosure Statement

No competing financial interests exist.

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